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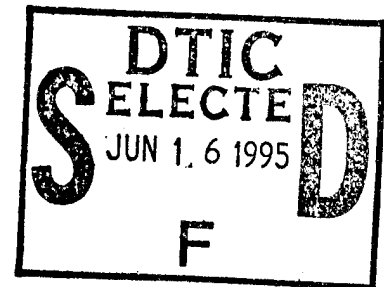
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Injuries of the mammalian central nervous system (CNS), which includes the axons of the retinal ganglion cells, result in axonal degeneration and irreversible loss of function. This is the reason for the failure to transplant neuroretina or other parts of the CNS, and also explains the permanent disablement resulting from injuries to the brain and spinal cord.

The use of low-energy helium-neon (He-Ne) laser irradiation has recently attracted attention concerning the treatment of nerve injury. Studies in the rat optic nerve have yielded several key observations which support the notion that treatment with low-energy laser is beneficial to injured rat optic nerves, provided that all parameters are well-calibrated and optimal. Below is a summary of our major results in two major aspects: (i) in delaying degeneration or rescuing marginally injured nerves; and (ii) in supporting regrowth when combined with an additional treatment modality.

## **EXPERIMENTAL SETUP**

### **1. OPTIMIZATION OF THE LOW-ENERGY LASER EFFECT IN REDUCING POSTTRAUMATIC DEGENERATION**

Irradiation employing a nonoptimal parameter could have either a devastating effect or no effect at all. Most of our studies have employed an electrophysiological setup in which measurements of compound action potential were carried out 2 weeks after injury. This time frame was chosen because at 2 weeks after injury the electrophysiological activity of the injured treated nerve itself is very low, so that any beneficial effect resulting from any treatment is detectable. Our studies involving calibration of the parameters are summarized below.

**1.1. Effects of low-energy He-Ne laser irradiation (10.5 mW, 1.1-mm beam diameter, 2 min, 10 consecutive days) on electrical activity of uninjured nerves**

Group	Number of rats	Irradiation mW    min		Action potential amplitude (μV)	Significance (P)
<hr/>					
Normal uninjured					
nonirradiated	232	—	—	4585.3 ± 1995.1	—
Uninjured					
irradiated	10	35	2	4850.0 ± 1415.2	P = 0.679

The results are expressed by the average ( $\mu$ V)  $\pm$  SD of the amplitudes of the action potentials. The lack of irradiation of uninjured normal nerves expressed by the P value according to Student's t-test.

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## 1.2. Beneficial effects of low-energy He-Ne laser irradiation on injured rat optic nerves as a function of:

### a. *Time of commencement of irradiation*

Group	Number of rats	Commencement of irradiation	Duration of irradiation	Action potential amplitude ( $\mu\text{V}$ ) (mean $\pm$ SEM)
A	8	immediate	1 week	1406.0 $\pm$ 225.0
B	5	2 h	1 week	810.0 $\pm$ 42.0
C	5	5 h	1 week	368.0 $\pm$ 100.0
D	4	24 h	1 week	390.0 $\pm$ 244.0
E	5	immediately preoperative	1 week	1700.0 $\pm$ 627.2
F	3	1 h preoperative	1 week	226.7 $\pm$ 25.2
G	3	30 min preoperative	1 week	166.7 $\pm$ 27.9

All groups were treated for 2 min at 10.5 mW. Groups A, B and E differ significantly from the group of injured untreated nerves, with  $P < 0.005$  (A, B) and  $P < 0.01$  (E). Groups C and D do not differ significantly from injured untreated nerves ( $505.5 \pm 303.5$  mW), having  $P$  values of 0.9303 and 0.8756, respectively. These results suggest that treatment is optimal when irradiation is commenced either immediately before injury or immediately after injury. The effect is considerably less beneficial when the first irradiation is delayed.

**b.**     *Number and frequency of treatments*

Group	Number of rats	Irradiation		Action potential amplitude ( $\mu$ V)	Significance (P)
		Days	Times/day		
A	14	7	1	$089.3 \pm 215.7$	$P < 0.001$
B	16	4	1	$1004.7 \pm 264.0$	$P < 0.001$
C	11	7	2	$336.4 \pm 130.6$	$P < 0.25$
D	11	4	2	$631.8 \pm 485.9$	$P < 0.25$
E	38	—	—	$505.5 \pm 303.5$	—

Irradiation (10.5 mW, 2 min) was initiated immediately after injury. The results are expressed by the average ( $\mu$ V)  $\pm$  SD of amplitudes of the action potentials. The significance of the difference from injured nonirradiated nerves ( $505.5 \pm 303.5$ ) is expressed by the P value of Student's t-test.

c. *Power and duration of each treatment*

Number of rats	Irradiation		Action potential amplitude ( $\mu$ V)	Significance (P)
	Power (mW)	Duration (min)		
10	35.0	2	1950.0 $\pm$ 1183.0	P < 0.000
25	10.5	2	1784.0 $\pm$ 717.5	P < 0.000
8	10.5	3	1950.0 $\pm$ 711.1	P < 0.000
4	10.5	5	nonrecordable	—
5	10.5	10	40.0 $\pm$ 89.4	P < 0.001
4	3.5	2	375.0 $\pm$ 150.0	P < 0.5
5	3.5	3	320.0 $\pm$ 175.4	P < 0.25
3	3.5	4	150.0 $\pm$ 86.6	P < 0.05
38	—	—	505.5 $\pm$ 303.5	—

Irradiation was initiated immediately after injury in all groups. The results are expressed by the average ( $\mu$ V)  $\pm$  SD of the amplitudes of the action potentials. The significance of the difference from injured nonirradiated nerves (505.5  $\pm$  303.5) is expressed by the P value of Student's t-test.



**d.**     *Wavelength of the laser*

Wavelength (nm)	Power (mW)	Number of animals	Action potential amplitude ( $\mu$ V)
632.8	8	7	$390 \pm 51$
514.0	8	8	$189 \pm 47$
476.0	8	8	$103 \pm 25$

All animals were subjected to daily irradiation for 2 min. The first irradiation was performed immediately after injury.

**1.3. Low-energy laser irradiation decreases an injury-induced deficit in metabolic activity**

*Effect of low-energy laser on metabolic activity.* The metabolic activity was monitored from the adult rat optic nerve by measuring changes in nicotinamide adenine dinucleotide (NADH) levels in response to anoxia (as was already discussed in details previously). The metabolic state was determined after nerve exposure and was continued to be measured after a crush injury was made at 1-h intervals up to 4 h postinjury. Following injury, as we have already reported, a reduction in the NADH response to anoxia was observed, reflecting a decrease in nerve metabolic activity. Two groups of animals – untreated ( $n = 13$ ) and laser irradiated ( $n = 11$ ) – were followed for 4 h after injury (Fig. 1). The injury-induced changes in the metabolic state of the tissue was significantly affected by the laser irradiation ( $F = 51.98$ ;  $P = 0.0001$ ). In the untreated group, 30 min after the injury NADH response to anoxia was  $37.3 \pm 3.9\%$ . However, in the treated animals a significant preservation of nerve metabolic activity was observed. Thirty minutes after injury, 15

min after laser irradiation, i.e., NADH response to anoxia was  $84.8 \pm 12.4\%$  of preinjury level. With increasing time the metabolic activity slightly decreases and stabilized on a statistically significant higher level than the untreated animals. Two hours postinjury was the only time point where no difference was found between the two groups.

Viability of neuronal tissue demands morphological integrity. When the integrity of a CNS axon is anatomically or morphologically interrupted, the distal segment of the axon which was disconnected from its cell body, degenerates (Wallerian degeneration). Consequently, the cell body fails to reestablish the contact with its end organ and, subsequently, degenerates as well. The posttraumatic degeneration delaying effect of the low energy laser irradiation can be potentially utilized to facilitate regeneration before irreversible degeneration and scarring sets in. This beneficial effect has been exploited successfully in treatments aiming at regeneration. More research is required to find ways to use the degeneration delaying effect of low energy laser in a combined treatment to induce regeneration.

#### **1.4. Discussion**

The results suggest that 2 weeks of daily He-Ne laser irradiation of 10.5 mW for 2 or 3 min, or of 35 mW for 2 min, leads to maximal delay of degeneration in moderately crushed optic nerves of rats. Irradiation lasting less than 2 min are less effective, while irradiation for longer than 3 min causes aggravation of the optic nerve damage. Irradiation twice a day for 4 or 7 days was not more effective than a repeated single daily irradiation, and did not delay degeneration of the injured nerve. Previous investigations have shown that the time lapse between injury and treatment is critical. Delay of degeneration is maximal when irradiation is initiated either immediately before or immediately after injury. Irradiation initiated 2 h after the crush was about half as effective as immediate irradiation, and no effect was observed when the laser was applied for the first time 5 h or more after the injury. We also showed that the same treatment, effective

when applied immediately prior to the injury, is significantly less effective when given 1.5 h or more before the injury. The duration of the treatment period is also important: treatment for 14 consecutive days was slightly more effective than treatment lasting 7 days, and significantly more effective than 4 days of irradiation.

According to our experiments, it seems that the 632.8 nm wavelength of the He-Ne laser is fortuitously suitable for inducing the desired effect of delaying the posttraumatic degeneration in our rat CNS model. This is probably a result of this wavelength being capable of penetrating behind the eyeball and absorbed in the appropriate chromophore, possibly in the glial cells. The results also demonstrated the dose-dependent effect of the laser irradiation with high doses, resulting either from long daily irradiation (5 min and 10 min), from high frequency (twice a day) being injurious, and lower doses (low power or short duration) being more effective. While the incoherent infrared irradiation failed to alter the course of the posttraumatic degeneration of the nerve, it is not conclusive that this wavelength is unsuitable for the purpose, since energy densities irradiated were much lower than that of the He-Ne laser irradiation.

## **2. REGENERATION IS BENEFICIAL FOLLOWING A TREATMENT COMBINED WITH LOW-ENERGY HE-NE LASER IRRADIATION**

### **2.1. Description of the experiments**

The experiments were performed on 42 adult rabbits (albinos, Weizmann Institute Animal House) 5–12-month old, weighing 2.2–3 kg each. The study included four groups (see Table e).

#### **a. *Surgery for injury of the optic nerve and implantation of conditioned medium***

Animals were anesthetized using 35 mg/kg ketamine and 6 mg/kg xylazine injected

intramuscularly.

The left optic nerve was exposed under a dissecting microscope, as was described previously, and injured at a distance of 4–6 mm from the eyeball by complete transection, using a sharpened dissecting needle. The meningeal membrane was intentionally partially spared to ensure continuity of the nerve and to permit placement and retention of the nitrocellulose film, described below. A nitrocellulose film, 2–3 mm long by 1 mm wide, was inserted at each site of injury. In the injured control group (Table e – B) and in the injured group, which received irradiation only (Table e – C), the nitrocellulose was soaked in serum-free medium (DMEM, Gibco) for 1 h and inserted into the lesion site. In the experimental group, which was treated by the combined method (Table e – D), the nitrocellulose was soaked in CM (100 mg protein/ml) for 1 h before being inserted into the lesion site.

**b.     *Preparation of conditioned medium***

Carp (*Cyprinus carpio*, 800–1200 g) were purchased from Tnuva, Israel. The fish were deeply anesthetized with 0.05% tricaine methanolsulfonate (Sigma). Optic nerves were crushed intraorbitally, as previously described and the fish replaced in their tanks. Eight days later, the fish were reanesthetized. The crushed regenerating nerves were dissected out, transferred into serum free medium (DMEM, Gibco) and incubated for 1 h at room temperature. CM was then collected and stored at –20°C. The protein content of the medium was determined by the Bradford method. The medium was diluted to 100 mg protein/ml. From this stock solution, 5–10 µl were used for each piece of nitrocellulose.

**c.     *Irradiation***

The He-Ne laser irradiated rabbits (Table e – C and D) were irradiated transocularly by a Spectra Physics Stabilite model 124-B He-Ne laser (632.8 nm, 35 mw). The laser beam, 1.1 mm in diameter, was directed at the pupil of the left eye. The first 5 min irradiation was given within

30 min after the injury. Repeated irradiations were given to groups C and D once a day, for 10 consecutive days.

d. *Electrophysiological measurements*

Eight weeks after injury, the optic nerves were removed for electrophysiological measurement. Lateral canthotomy was performed on deeply anesthetized animals. The skin was removed from the rabbit skull; the skull opened with a rongeur and the bone between the eyes was removed; the brain was then displaced from above the orbits and the intracranial portion of the optic nerves were exposed from the optic foramen to the optic chiasm. Using the rongeur, the orbital roof was then removed, so that the whole length of the optic nerve from the eyeball to the chiasm could be taken out. After the conjunctiva and the muscles from the eyeball were dissected away starting from the limbus, the eyeball was elevated with the optic nerve which was then transected from the chiasm. The inevitable bleeding during this procedure caused considerable difficulty during surgery. The optic nerve, still connected to the eyeball, was immersed in fresh cold Krebs solution (125 mM NaCl; 5 mM KCl; 1.2 mM  $\text{KH}_2\text{PO}_4$ ; 26 mM  $\text{NaHCO}_3$ ; 0.6 mM  $\text{MgSO}_4$ ; 2.4 mM  $\text{CaCl}_2$ ; 10 mM D-glucose, aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ). While in the solution, the tissues surrounding the optic nerve were removed and the nerve was cut from the eyeball. After 1 h of recovery in the cold Krebs solution, the nerves were transferred to a vial of warmed ( $37^\circ\text{C}$ ) Krebs solution where cleaning of the optic nerve was completed, using a dissecting microscope. The nerve ends were then connected to two Ag-AgCl suction electrodes immersed in the bathing solution. A stimulating pulse was given through the electrode on one end, and the action potential was recorded on the other end. A Grass SD-9 stimulator was used for electrical stimulation (4 volts, 50  $\mu\text{sec}$ ). The signal was transmitted to a Medelec KS-7 preamplifier and then to an AA-7-T amplifier and a Medelec KS-7 electromyograph. The electrodes, stimulator and amplifier had a common grounding. The average amplitude of eight recorded potentials was photographed with a Polaroid camera.

## 2.2. Results

Nerves from three control groups and one experimental group were excised and measured electrophysiologically using the suction electrodes. Two parameters were measured: the amplitude of the action potential and its delay period.

Uninjured control nerves (Table e – A) were subdivided into two groups: (i) optic nerves of naive untreated animals, whose mean action potential was  $1676 \pm 859 \mu\text{V}$  ( $\pm$  SD;  $n = 13$ ) [Table f – A(a)]; and (ii) right optic nerves of animals, belonging to groups B-D in Table e, whose left optic nerves were treated. This group mean action potential was  $1824 \pm 1009 \mu\text{V}$  ( $\pm$  SD;  $n = 19$ ) [Table f – A(b)]. The mean compound action potentials of these two subgroups were not different. Measurements of compound action potentials of injured animals belonging to all operated groups was carried out 8 weeks after injury. All measurements of the injured controls resulted in values which were below detectable levels (Table f – B). The same results were obtained from injured nerves which received irradiation only (Table f – C). In group D, which included 17 experimental animals, injured and treated with the combination of laser irradiation and the soluble substances derived from the regenerating fish optic nerves, different results were obtained. In five out of 17 examined animals, a positive, though low, compound action potential was measured (20–30  $\mu\text{V}$ ). This activity was further characterized by a long delay period (mean = 1.7 msec) (Table f – D), which is significantly longer ( $P < 0.01$ ) than of the control groups.

## 2.3. Discussion

We have previously shown that treatment of injured adult rabbit optic nerves, with low energy He-Ne laser irradiation combined with application of soluble substances derived from regenerating fish optic nerves, leads to growth of axons within their own degenerative environment. In the present study we show that these axons are electrophysiologically active,

which provides further support to the notion that they are newly growing axons.

We showed in our previous work that in injured adult rabbit optic nerves treated with low energy He-Ne laser and soluble substances derived from regenerating fish optic nerves, there are abundant unmyelinated and thinly myelinated axons, which traverse the site of injury and extend a significant distance distally. We provided morphological and immunocytochemical evidences that these are newly growing axons, originating from the retinae and embedded in CNS glial cells. In the present study, five out of the 17 treated nerves exhibited electrophysical activity. It is possible that more than five were active but that the others escaped detection; the nerve segments used for measurements included parts of the nerve both proximal and distal to the site of injury. There may have been cases in which successfully growing nerves traversed the site of injury but did not extend as far as where the recording electrode was placed. With the available data we can conclude that at least five nerves were electrophysiologically active.

The low compound action potential of the five electrophysiologically active nerves of the experimental group is reasonable, considering the small number of viable axons which one can expect to be at the far distal region of the treated nerves. In previous studies, we observed that about 5% of the number of axons in a normal uninjured nerve traversed the site of injury and were present 2 mm distal to the site of injury. The number of such axons gradually decreased upon increasing distance from the site of injury. As normal nerves gave a mean compound action potential of 1600–1800  $\mu$ V, one could not expect more than was found in the treated nerves which had, at the most, 5% of the axons present in an intact nerve. The uniqueness of these axon compound action potentials, however, is in their prolonged delay time period, which is typical of unmyelinated and thinly myelinated axons, and not heavily myelinated axons, indicating that these are not spared axons but newly growing axons.

These results provide encouragement that the approach taken in this study, of induction of growth of axons within their own environment, is a valid one. Further studies are currently being carried out using *in vivo* electrophysiological recordings.

### 3. CONCLUSIONS

Low-energy laser irradiation reportedly has various effects on cells, tissues and organs, manifested in biochemical, morphological and physiological phenomena and alteration of growth and regenerative parameters. The effects seem to be particularly pronounced in the hematological and immune systems, and on connective and neural tissues. There are only a few reports of the biochemical and morphological effects of low-level irradiation on the neural system. Direct or indirect irradiation of the rat brain caudate nucleus by either He-Ne or nitrogen ultraviolet laser caused significant changes in the concentrations of various monoamines and amino acids in the corpus striatum. Irradiation of mice retina with a 0.5 mW laser caused an increase in protein synthesis and shedding of photoreceptors in the outer segment and in the interreceptor matrix. Laser irradiation stimulated neurons directly in animals, as well as in humans. Other irradiation regimens, however, did not influence human nerves. The neural effects of low-energy irradiation has been exploited to relieve pain and clonus in various disease states, although some of these results could not be confirmed.

In our model, the primary events induced by the laser irradiation probably occurred in the optic nerve rather than in the retinal cell bodies, although the laser beam traversed the entire eye. This assumption is based on the fact that the diameter of the irradiation beam on the retina is very small, because of its focusing by the refractive media of the eye. The red light of the He-Ne laser penetrates a few millimeters behind the eye, and its scatter in the retrobulbar tissues accounts for diffuse irradiation of the optic nerve, including the crush site and its vicinity. In an analogous situation, direct transcutaneous irradiation of the damaged region behind the eye of rabbits, after orbitotomy, yielded histological results similar to those obtained following irradiation via the eye. If low-energy laser irradiation is employed clinically as part of the treatment of optic nerve injuries, it will not be performed transocularly since the energy required is higher than the threshold for retinal injury. It will have to be performed transorbitally, possibly via a fiber-optic light guide,



with or without orbitotomy.

The mechanism underlying this effect is poorly understood. The most reasonable hypothesis is that the low energy laser, which can penetrate only few millimeters of tissue, is absorbed by chromophores specific to each wavelength, without an appreciable rise in overall tissue temperature. When these chromophores are enzymes, organelles or cell membrane molecules, their activity is altered and, thus, the metabolism or membrane permeability to specific molecules are altered. The action spectra required to prove that this is a photochemical phenomenon has been demonstrated for enzymes.

In our model, the primary events induced by the laser irradiation probably occurred at the optic nerve rather than the retinal cell bodies, although the laser beam traversed the entire eye. This assumption is based on the fact that the diameter of the irradiation beam on the retina is very small, due to its focusing by the refractive media of the eye. The light of the He-Ne laser penetrates a few millimeters behind the eye, and its scatter in the retrobulbar tissues accounts for diffuse irradiation of the optic nerve, including the crush site and its vicinity. Furthermore, direct irradiation of the damaged region through the skin behind the eye of rabbits, after orbitotomy, yielded similar histological results like those caused by irradiation via the eye.

**Table e.**     *Treatments applied*

Group	Treatment	Number of animals
A	Normal uninjured control	32*
B	Injured untreated control	8
C	Injured irradiated only	4
D	Combined treatment	17

\*Of the 32 nerves, 13 were the right optic nerves of animals whose left optic nerves were injured and untreated or injured and treated. The electrophysiological activity of these nerves was not different from that of uninjured animals.

**Table f.** *Electrophysiological activity of axons in fully-treated injured (D), in injured untreated (B), in partially treated (C) and in normal nerves (A, B-D)*

Group	Treatment	Compound action potential (mean) ( $\mu$ V)	Delayed response time (mean) (msec)
A	Intact nerve	1823.6 $\pm$ 1008.6 (n = 19)	0.42 $\pm$ 0.12
B-D	Contralateral (intact)	1676.0 $\pm$ 858.9 (n = 13)	0.52 $\pm$ 0.18 (a)*
B	Injured untreated (control)	Undetectable (n = 12)	
C	Injured irradiated only	Undetectable (n = 4)	
D	Injured treated	20-30 (5 out of 17)	1.72 $\pm$ 0.45 (b)

\*(a) Significantly differs from (b) ( $P < 0.01$ ). Full treatment consists of low-energy laser irradiation and application of soluble factors from growing nerves (CM).

## PUBLICATIONS EMERGED FROM THIS WORK

1. Rosner, M., Caplan, M., Cohen, S., Duvdevani, R., Solomon, A., Assia, E., Belkin, M. and Schwartz, M. (1993) Dose and temporal parameters in delaying injured optic nerve degeneration by low-energy laser irradiation. *Lasers Med.* (in press).
2. Schwartz, M. (1993) Beneficial effect of low-energy helium-neon laser on the injured adult rat optic nerve. In: *Progress in Biomedical Optics, Vol. 1883, Low-Energy Laser Effects on Biological Systems* (Schwartz, M., Belkin, M. and Katzir, A., eds.) SPIE, pp. 2-7.
3. Eitan, S., Solomon, A., Yoles, E., Lavie, V., Hirschberg, D.L., Kenan, G., Belkin, M. and Schwartz, M. (1993) Regeneration of mammalian optic nerves and recovery of visual evoked potential response by treatment with nerve-derived transglutaminase. *Science* (submitted).